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13. ABSTRACT *(Maximum 200 words)*

Healthy male and female human volunteers were exposed to 50 ppm or 100 ppm trichloroethylene (Tri) by inhalation for 4 h. Blood and urine samples were taken at various times before, during, and after the exposure period for analysis of glutathione (GSH), related thiols and disulfides, and GSH-derived metabolites of Tri. The GSH conjugate of Tri, S-(1,2-dichlorovinyl)glutathione (DCVG), was found in the blood of all subjects from 30 min after the start of the 4-h exposure to Tri to 1 to 8 h after the end of the exposure period, depending on the dose of Tri and the sex of the subject. male subjects exposed to 100 ppm Tri exhibited a maximal content of DCVG in the blood at 2 h after the start of the exposure of 46.1 $\pm$ 14.2 nmol/ml (n=8), whereas female subjects exposed to 100 ppm Tri exhibited a maximal content of DCVG in the blood at 4 h after the start of the exposure of only 13.4 $\pm$ 6.6 nmol/ml (n=8). Pharmacokinetic analysis of blood DCVG concentrations showed that the area under the curve value was 3.4-fold greater in males than in females, while the t1/2 values for systemic clearance of DCVG were similar in the two sexes. Analysis of the distribution of individual values indicated a possible sorting, irrespective of gender, into a high- and a low-activity population, which suggests the possibility of a polymorphism. The mercapturates N-acetyl-1,2-DCVC and N-acetyl-2,2-DCVC were only observed in the urine of 1 male subject exposed to 100 ppm Tri. Higher contents of glutamate were generally found in the blood of females, but no marked differences between sexes were observed in contents of cyst(e)ine or GSH or in GSH redox status in the blood. Urinary GSH output exhibited a diurnal variation with no apparent sex- or Tri exposure-dependent differences.

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IDENTIFICATION OF *S*-(1,2-DICHLOROVINYL)GLUTATHIONE IN THE BLOOD OF HUMAN VOLUNTEERS EXPOSED TO TRICHLOROETHYLENE

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fold greater in males than in females, while the $t_{1/2}$ values for systemic clearance of DCVG were similar in the two sexes. Analysis of the distribution of individual values indicated a possible sorting, irrespective of gender, into a high- and a low-activity population, which suggests the possibility of a polymorphism. The mercapturates N-acetyl-1,2-DCVC and N-acetyl-2,2-DCVC were only observed in the urine of 1 male subject exposed to 100 ppm Tri. Higher contents of glutamate were generally found in the blood of females, but no marked differences between sexes were observed in contents of cyst(e)ine or GSH or in GSH redox status in the blood. Urinary GSH output exhibited a diurnal variation with no apparent sex- or Tri exposure-dependent differences. These results provide direct, in vivo evidence of GSH conjugation of Tri in humans exposed to Tri and demonstrate markedly higher amounts of DCVG formation in males, suggesting that their potential risk to Tri-induced renal toxicity may be greater than that of females.

Trichloroethylene (Tri; also known as trichloroethene) is a colorless, nonflammable organic solvent that is used in numerous industrial processes and is a major surface and ground water contaminant. Tri has been identified as a carcinogen in rodents, although the susceptibility to and target organ specificity for Tri exhibits sex- and species-dependent differences (Davidson & Beliles, 1991; Goeptar et al., 1995). Although lung and liver tumors have been found in male mice, kidney tumors have been found in male rats. Because of its widespread use and presence in the environment, there is considerable concern about the potential risk to humans from occupational or environmental exposure to Tri. In fact, the International Agency for Research on Cancer (IARC) recently reclassified Tri as a Group 2A chemical, which means that it considered Tri to be "probably carcinogenic in humans: limited human evidence, sufficient evidence in experimental animals" (IARC, 1995). The primary human data from Tri exposure include a slight increase in liver and biliary tract tumors and non-Hodgkin's lymphoma (Davidson & Beliles, 1991).

The possibility of the kidney as a target organ for Tri in humans has been a subject of considerable debate in recent years. Although several studies have indicated either acute nephrotoxicity and kidney dysfunction from occupational Tri exposure (Davis et al., 1989; Nagaya et al., 1989; Rasmussen et al., 1993), others failed to observe increases in urinary parameters that indicate renal injury and concluded that Tri is not nephrotoxic in humans at low exposure levels (Selden et al., 1993). Three cohort studies (Anttilla et al., 1995; Axelson et al., 1994; Spirtas et al., 1991) found no evidence of increased kidney cancer risk in workers exposed to Tri. In contrast, a study of German cardboard factory manufacturing workers (Henschler et al., 1995a) observed five cases of kidney cancer in exposed workers as compared with none in the control population. This study, however, caused significant controversy, and it was claimed that the incidences of renal cancer were a cluster and that it was not a valid epidemiological study (Bloemen & Tomenson, 1995; Henschler et al., 1995b; Swaen, 1995). Brüning et al. (1998) obtained urine and blood samples from a 17-yr-old male who ingested approximately 70 ml of Tri in a suicide attempt. Although the patient did not exhibit any of the stan-

dard clinical parameters of nephrotoxicity, analysis of urinary proteins was consistent with tubular damage, demonstrating that a single, oral dose of Tri can produce acute nephrotoxicity in humans. The uncertainty about the risk to humans suggests that further study is warranted.

Tri-induced toxicity and carcinogenicity are dependent on its metabolism, which occurs by either an oxidative pathway, with the initial step catalyzed by cytochrome P-450 (P-450), or glutathione (GSH) conjugation, catalyzed by GSH *S*-transferases (GSTs) (Davidson & Beliles, 1991; Goeptar et al., 1995). The oxidative pathway generates chloral hydrate, trichloroethanol, tri- and dichloroacetate, and trichloroethanol glucuronide as major metabolites. Some of these metabolites have been associated with hepatotoxicity and liver tumors in rodents (Larson & Bull, 1992a, 1992b) or lung tumors in mice (Forkert & Birch, 1989; Green et al., 1997a; Odum et al., 1992). GSTs form *S*-(1,2-dichlorovinyl)glutathione (DCVG) as the initial metabolite, and subsequent metabolites of DCVG are associated with nephrotoxicity in rats (Elfarra et al., 1986) and acute cytotoxicity in isolated proximal tubular cells from rat kidneys (Lash & Anders, 1986).

The initial reaction of the GSH conjugation pathway for Tri can occur in either the liver or kidneys (Lash et al., 1995, 1998). Although the kidney is the primary target organ for reactive metabolites of this pathway, the liver is quantitatively the predominant site of DCVG formation. As with other GSH *S*-conjugates, DCVG undergoes interorgan metabolism and is further processed to the cysteine conjugate *S*-(1,2-dichlorovinyl)-L-cysteine (DCVC) or the corresponding mercapturates, *N*-acetyl-*S*-(1,2-dichlorovinyl)-L-cysteine (NAc-1,2-DCVC) or *N*-acetyl-*S*-(2,2-dichlorovinyl)-L-cysteine (NAc-2,2-DCVC), by γ-glutamyltransferase (GGT), dipeptidases, and cysteine conjugate *N*-acetyltransferase (cys-NAT) in biliary, small intestinal, or renal epithelia (Lash et al., 1988). Metabolites of this pathway accumulate in proximal tubular cells of the kidneys due to the tissue distribution of enzymes and transport carriers.

Formation of DCVC represents a branch point in the GSH-dependent metabolism of Tri as one of two alternative pathways can occur, a detoxication reaction to form NAc-1,2-DCVC or NAc-2,2-DCVC, catalyzed by cys-NAT, or a bioactivation reaction to form a reactive thiol *S*-(1,2-dichlorovinyl)thiol (DCVSH), catalyzed by the cysteine conjugate β-lyase (β-lyase) (Elfarra et al., 1986; Lash et al., 1986). DCVSH can rearrange to form a potent acylating species that forms covalent adducts with proteins and DNA, thereby producing renal cellular injury. Metabolites produced after administration of DCVG or DCVC are mutagenic in the Ames test (Commandeur et al., 1991; Dekant et al., 1986a; Vamvakas et al., 1988a). Additionally, more direct evidence of DCVC-induced genotoxicity was obtained by Vamvakas et al. (1988b, 1989), who observed unscheduled DNA synthesis and micronucleus formation in Syrian hamster fibroblasts and unscheduled DNA synthesis in a renal epithelial cell line. The presence of

the β-lyase pathway in human kidney (Lash et al., 1990) is consistent with the possibility that these same mechanisms leading to Tri-induced nephrotoxicity and nephrocarcinogenesis can occur in humans.

Evidence for the occurrence of the GSH conjugation pathway in vivo in rodents (Bernauer et al., 1996; Birner et al., 1993; Commandeur & Vermeulen, 1990; Dekant et al., 1986b) and humans (Bernauer et al., 1996; Birner et al., 1993) exposed to Tri has been obtained by measurement of the stable, detoxication products, NAc-1,2-DCVC and NAc-2,2-DCVC, in urine. Recovery of mercapturates relative to oxidative metabolites of Tri in urine is very small, however, with ratios of mercapturate to trichloroethanol and trichloroacetate of 1:100 to as low as 1:4000. Hence, it has been argued that flux of Tri through the GSH conjugation pathway represents an extremely minor pathway of Tri metabolism, particularly in humans, and that formation of reactive metabolites from Tri by the GSH conjugation pathway occurs at too low a rate to produce toxicity in humans (Goeptar et al., 1995; Green et al., 1997b).

In the present study, healthy male and female human volunteers were exposed to 50 or 100 ppm Tri by inhalation. Blood and urine samples were collected over a period of several days after the 4-h exposure to analyze for metabolites of Tri. Here, we report results from measurements of GSH and GSH-derived metabolites. A pharmacokinetic analysis of P-450-derived metabolites of Tri in urine and blood of these same volunteers is presented in a separate publication (Fisher et al., 1998). Formation of DCVG was observed in the blood of all subjects. DCVG levels were higher in subjects who were exposed to the higher dose of Tri, and peak blood levels of DCVG were approximately twofold higher in males than in females. Urinary GSH content showed considerable variation between subjects and over time. This is the first demonstration of recovery of DCVG in humans exposed to Tri at subtoxic doses.

MATERIALS AND METHODS

Materials

GSH, L-γ-glutamyl-L-glutamate, GGT, iodoacetic acid, and 1-fluoro-2,4-dinitrobenzene were purchased from Sigma Chemical Co. (St. Louis, MO). DCVG was a gift from Dr. Adnan A. Elfarra (University of Wisconsin, Madison, WI) and was synthesized by reacting GSH with Tri in sodium and liquid ammonia as described previously (Elfarra et al., 1986). Purity of DCVG (>95%) was determined by high-performance liquid chromatography (HPLC) analysis, and identity was confirmed by proton nuclear magnetic resonance (NMR) spectroscopy. NAc-1,2-DCVC and NAc-2,2-DCVC were synthesized by the same method as for DCVG, except that Tri was reacted with *N*-acetyl-L-cysteine instead of GSH. Purity of NAc-1,2-DCVC and NAc-2,2-DCVC (>95%) was determined by gas chromatog-

raphy/mass spectrometry (GC/MS), and identity was confirmed by proton NMR spectroscopy. All other chemicals were of the highest purity available and were obtained from commercial sources.

Exposure of Subjects to Tri

Four-hour Tri inhalation vapor exposures were carried out at Research Triangle Institute, Human Studies Facility, Research Triangle Park, NC. The whole-body exposures were conducted in a chamber as described previously (Raymer et al., 1993). The human use research protocol was approved by the Research Triangle Institute Institutional Review Board and the U.S. Army Human Studies Research Review Board. Female (10) and male (11) volunteers, ranging in age from 18 to 36 yr, were selected for this study after physical examination. Two individuals were exposed simultaneously to either 50 ppm or 100 ppm Tri vapor while sitting in a chamber for 4 h in the morning. Exposures began at approximately 9:00 a.m. Sleeping cots were provided for the volunteers while they remained in the chamber over the next 18 h, which they did except to use the bathroom. Several blood samples were taken from each individual by venous catheter during the exposure period and for 18 h postexposure. Alveolar breath samples were collected from a few of the exposed volunteers after the 4-h exposure using methods described by Pleil and Lindstrom (1995). After this period, the volunteers returned to the institute once each day for 3 d to deliver their voided urine and to have blood drawn by venipuncture.

Blood and Urine Sample Collection and Processing

A portion (1 ml) of blood and urine samples was placed in glass test tubes containing 0.2 ml of 70% (v/v) perchloric acid for analysis of GSH, related thiols and disulfides, and DCVG, and was frozen until analysis. A portion of urine samples was directly frozen until analysis of mercapturates.

HPLC Analysis of GSH, Related Thiols and Disulfides, and DCVG

After thawing blood and urine samples, 0.1 ml of a solution containing 15 m*M* bathophenanthroline disulfonate (antioxidant) and 1.5 m*M* L-γ-glutamyl-L-glutamate (internal standard) was added, and samples were mixed and were centrifuged to remove protein. Acid-soluble supernatants were then derivatized with iodoacetic acid and 1-fluoro-2,4-dinitrobenzene for analysis of *S*-carboxymethyl-*N*-dinitrophenyl GSH and *N*-dinitrophenyl DCVG by ion-exchange HPLC as described previously (Fariss & Reed, 1987; Lash & Jones, 1985a). Other compounds that were quantitated in blood included the *N*-dinitrophenyl derivative of L-glutamate, the *S*-carboxymethyl-*N*-dinitrophenyl derivative of L-cysteine, and the *N,N*-bis-dinitrophenyl derivative of glutathione disulfide (GSSG) and the mixed disulfide of GSH and L-cysteine (CySSG). Derivatives were detected by absorbance at 365 nm and were quantitated by electronic integration of peaks and comparison with authentic standards. The limit of detection

was 50 pmol, a linear detector response was obtained with samples containing from 50 pmol up to 10 nmol, and the efficiency of derivatization was estimated to be 85% to 110%, with a correction made for this variation by use of an internal standard.

Besides comparison of retention time with that of the derivative of authentic DCVG standard, the identity of the DCVG peak was confirmed as done previously (Lash & Jones, 1985a) by a 15-min treatment of selected samples with 1 U of a partially purified preparation of GGT that also contains dipeptidase activity (data not shown). As a consequence of this treatment, DCVG was converted to DCVC, whose *N*-dinitrophenyl derivative is also detected by this HPLC method (Lash & Anders, 1989).

GC/MS Analysis of NAc-1,2-DCVC and NAc-2,2-DCVC

Mercapturates of Tri (both isomers) were measured in urine samples as follows. Urine samples (4.0 ml) were treated with 0.5 ml of 10% (v/v) sulfuric acid, and acid-soluble supernatants were extracted with 3 × 2 ml diethyl ether. After addition of ethereal diazomethane to the combined ether extracts, samples were evaporated to dryness under a stream of nitrogen gas and were reconstituted in 0.25 ml ethyl acetate. Samples were analyzed via GC/MS with selected ion monitoring (*m/z* 144, 137, 88, 89) using an HP-5 column (30 m × 0.25 mm). Chromatography conditions were: injector temperature 175°C, initial temperature 60°C, initial time 2 min, final temperature 165°C, rate 20°C/min, final time 22.75 min, carrier gas He, pressure 1 psi. The limit of detection was 50 ng/ml.

Pharmacokinetic Analysis of Blood DCVG Data

Pharmacokinetic parameters for the blood concentration data of DCVG formation from subjects exposed to 100 ppm Tri were calculated as described by Medinski and Klaassen (1996). Half-life ($t_{1/2}$) values for Tri elimination were calculated from plots of the natural log of DCVG concentration versus time and area under the curve (AUC) was calculated with data from 0 h to 12 h postexposure.

Data Analysis

All values are means ± SE of measurements made on the indicated number of samples from separate individuals, except where indicated. Where appropriate, significant differences between means for data were first assessed by a one-way or two-way analysis of variance. When significant *F*-values were obtained, the Fisher's protected least significance *t*-test was performed to determine which means were significantly different from one another, with two-tail probabilities $<.05$ considered significant.

RESULTS

The GSH conjugate of Tri, DCVG, was readily detected in samples of the blood of male and female volunteers exposed by inhalation for 4 h to

100 ppm Tri. An illustration of this is given by the HPLC chromatograms in Figure 1, which show the presence of a distinct, large peak with retention time between that for the derivatives of glutamate and cystine in a blood sample from an individual exposed to Tri for 2 h (Figure 1B). This peak is clearly absent in a blood sample taken from the same individual prior to the Tri exposure (Figure 1A).

(A)

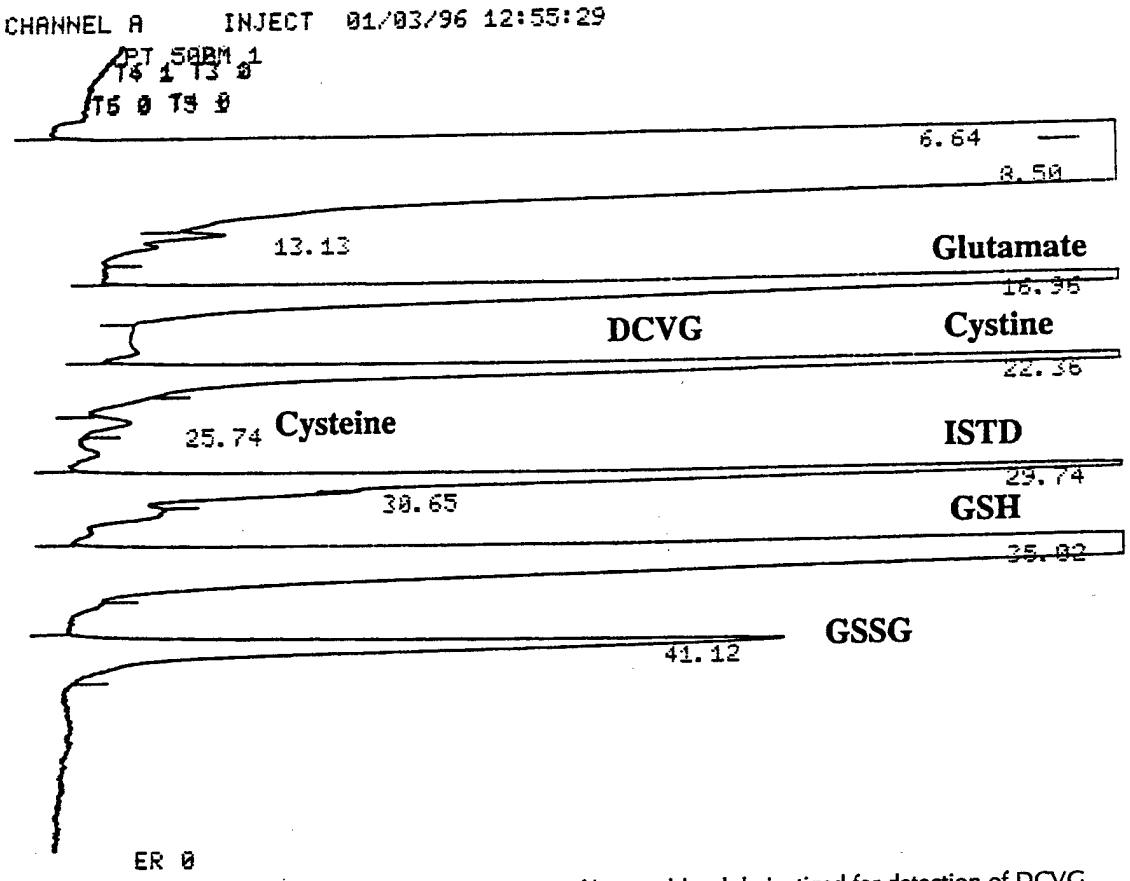

FIGURE 1. Representative HPLC chromatograms of human blood derivatized for detection of DCVG. Blood samples were deproteinated with perchloric acid, treated with bathophenanthroline disulfonate as an antioxidant and with L-γ-glutamyl-L-glutamate as an internal standard (ISTD), and acid extracts were derivatized with iodoacetic acid and 1-fluoro-2,4-dinitrobenzene as described in Materials and Methods. *N*-Dinitrophenyl derivatives of glutamate, DCVG and the ISTD, *N,N*-bis-dinitrophenyl derivatives of cystine and GSSG, and *S*-carboxymethyl-*N*-dinitrophenyl derivatives of cysteine and GSH were separated on an amine column by gradient HPLC using a methanol–acetate solvent system. Derivatives were detected by absorbance at 365 nm and were quantitated by integration with respect to derivatives of authentic standards. (A) HPLC chromatogram of blood from one individual at time 0 before Tri exposure. It is important to note that retention times will vary and decrease with use of the column because of derivatization of amine groups on the column by unreacted 1-fluoro-2,4-dinitrobenzene. This is compensated for by adjusting the concentration of acetate in the solvent.

(B)

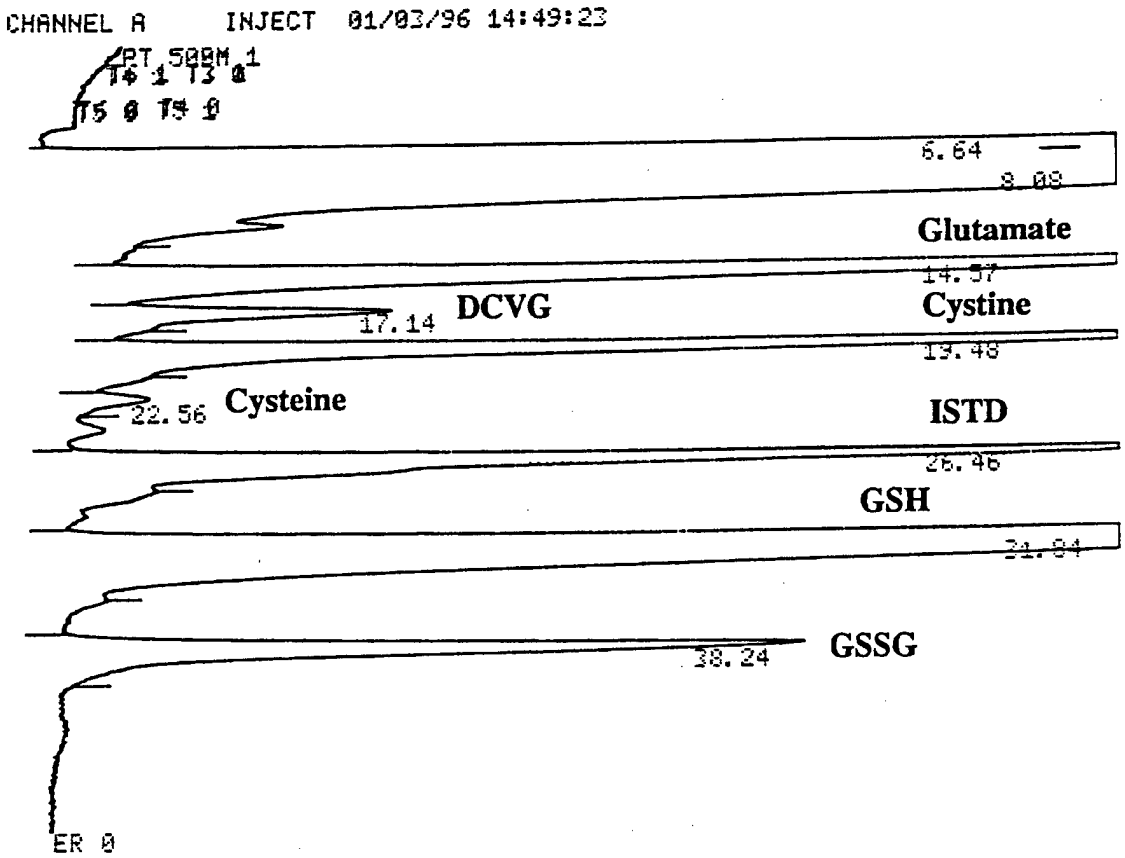

FIGURE 1. (*Continued*) Representative HPLC chromatograms of human blood derivatized for detection of DCVG. Blood samples were deproteinated with perchloric acid, treated with bathophenanthroline disulfonate as an antioxidant and with L-γ-glutamyl-L-glutamate as an internal standard (ISTD), and acid extracts were derivatized with iodoacetic acid and 1-fluoro-2,4-dinitrobenzene as described in Materials and Methods. *N*-Dinitrophenyl derivatives of glutamate, DCVG and the ISTD, *N,N*-bis-dinitrophenyl derivatives of cystine and GSSG, and *S*-carboxymethyl-*N*-dinitrophenyl derivatives of cysteine and GSH were separated on an amine column by gradient HPLC using a methanol–acetate solvent system. Derivatives were detected by absorbance at 365 nm and were quantitated by integration with respect to derivatives of authentic standards. (B) HPLC chromatogram of blood from that same individual as in (A) at 2 h after inhalation exposure to 100 ppm Tri. It is important to note that retention times will vary and decrease with use of the column because of derivatization of amine groups on the column by unreacted 1-fluoro-2,4-dinitrobenzene. This is compensated for by adjusting the concentration of acetate in the solvent.

The GSH conjugate of Tri, DCVG, was detected in the blood of males and females exposed to the lower dose (50 ppm) of Tri (Figure 2). Both of the females that were exposed to 50 ppm Tri exhibited maximal blood contents of DCVG of approximately 2.5 nmol/ml. Maximal blood content of DCVG was reached within 1 h of the start of the exposure, and DCVG was undetectable after 12 h (8 h after end of Tri exposure) in 1 individual and after 5 h (1 h after end of Tri exposure) in the other individual. The

pattern of appearance of DCVG in the blood of males exposed to 50 ppm Tri was generally similar to that in females. However, while 1 male subject had similar contents of DCVG in the blood (2.6 nmol/ml maximum), the other 2 male subjects had maximal contents of DCVG in the blood (25 to 30 nmol/ml) that were 10-fold higher than those in the female subjects.

The difference in detection of DCVG in the blood between males and females was more evident in the subjects exposed to the higher dose (100 ppm) of Tri (Figure 3). In this case, eight males and eight females were exposed, making statistical analysis possible. Although there was some variation among subjects, DCVG content in blood was clearly and significantly higher in males than in females (Figure 3A). Maximal content of DCVG in the blood of males (46.1 ± 14.2 nmol/ml) was more than threefold higher than the maximal content of DCVG in the blood of females

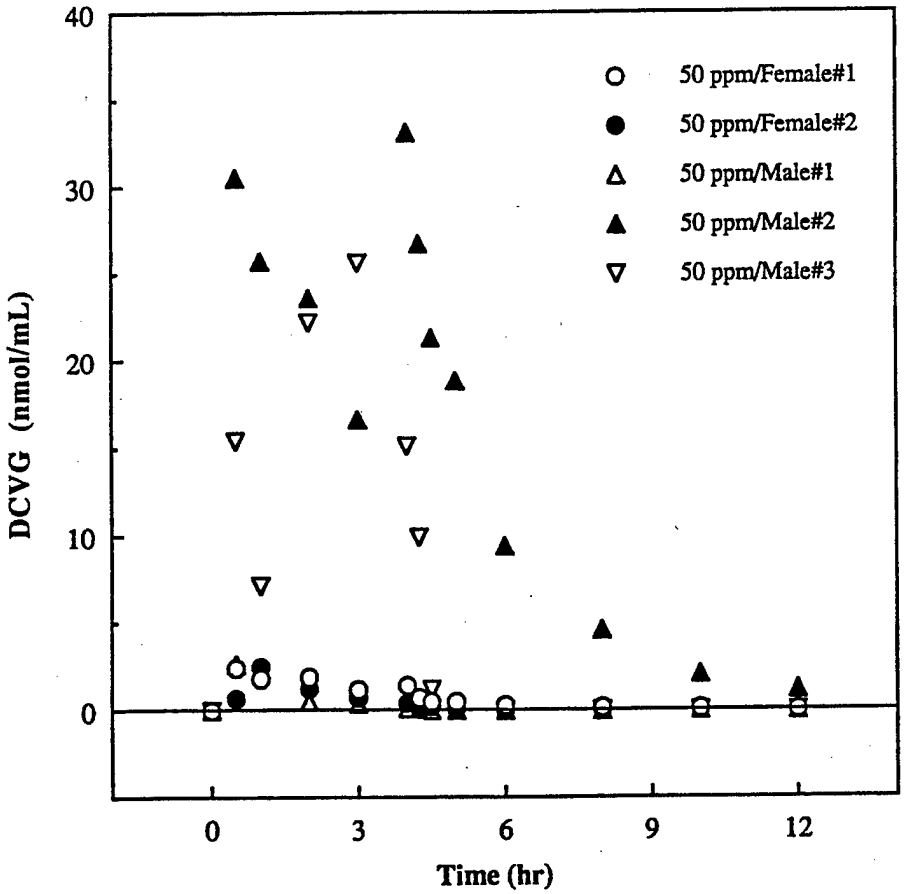

FIGURE 2. Content of DCVG in the blood of males and females exposed to 50 ppm Tri. Three male and 2 female volunteers were exposed to 50 ppm Tri for 4 h. Blood was sampled at the indicated times, where time 0 is the beginning of the exposure period. DCVG content in blood was determined by derivatization of perchloric acid extracts with 1-fluoro-2,4-dinitrobenzene and analysis by ion-exchange HPLC. Each point represents a value from one individual at the specified time.

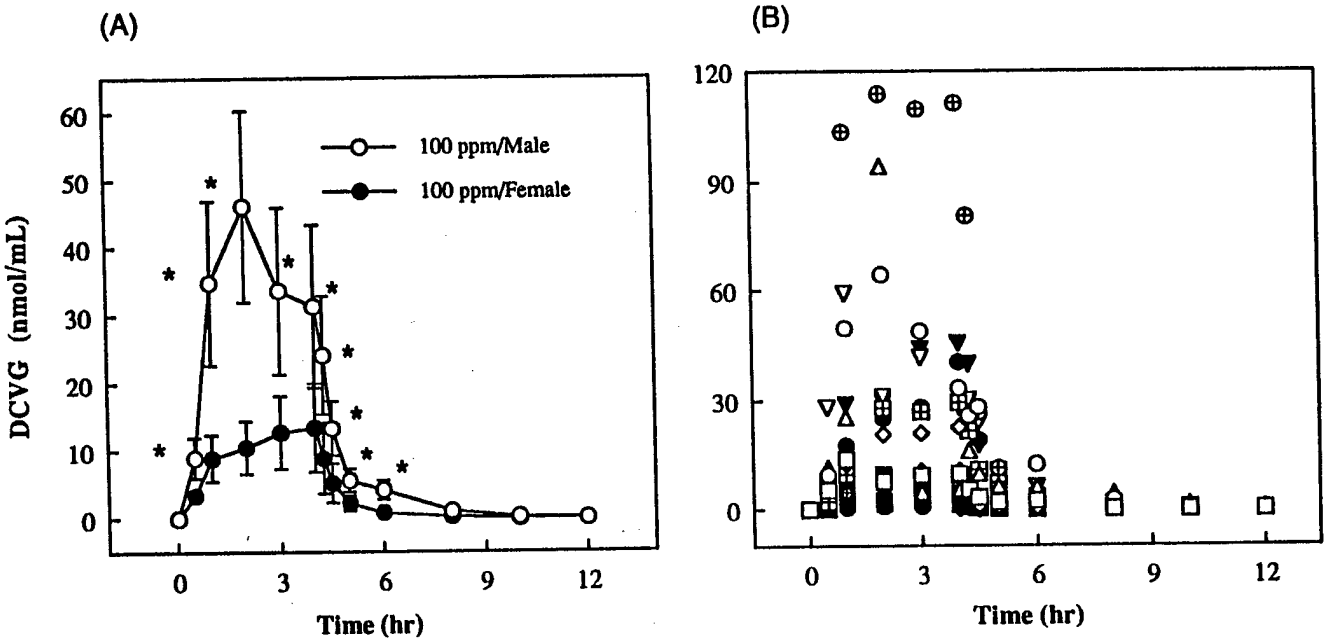

FIGURE 3. Content of DCVG in the blood of males and females exposed to 100 ppm Tri. Eight male and 8 female volunteers were exposed to 100 ppm Tri for 4 h. Blood was sampled at the indicated times, where time 0 is the beginning of the exposure period. DCVG content in blood was determined by derivatization of perchloric acid extracts with 1-fluoro-2,4-dinitrobenzene and analysis by ion-exchange HPLC. (A) Results are the means ± SE of measurements from eight subjects. Asterisk indicates significantly different ($p < .05$) from the corresponding sample from females. (B) Results are individual values from each of the eight male and eight female subjects.

(13.4 ± 6.6 nmol/ml). Time course differed somewhat between males and females as well, with maximal DCVG content being reached by 2 h after the start of Tri exposure in all males and by 4 h after the start of Tri exposure in all females. Individual values for DCVG content in blood from each of the eight males and eight females are also shown (Figure 3B) to assess whether there is a sorting of values according to something other than just gender, such as the existence of a genetic polymorphism in the GST or GSTs that catalyze the conjugation of Tri with GSH. Although most of the male subjects exhibited higher values of DCVG in the blood than the majority of the female subjects, half of the male subjects exhibited values of DCVG in the blood that were similar to or lower than those of the female subjects, suggesting that a polymorphism in GSH conjugation of Tri may exist. The cysteine conjugate of Tri, DCVC, was not detected in the blood of any of the subjects at either dose of Tri.

A pharmacokinetic analysis of DCVG formation and elimination in the blood at the 100 ppm exposure dose of Tri was conducted, using the data in Figure 3A (Table 1). DCVG reached peak concentrations during vapor exposure to Tri and achieved blood concentrations of DCVG were greater in males than in females exposed to either 50 or 100 ppm Tri (Figures 2 and 3). The AUC value for DCVG blood concentration was 3.4-fold greater in males as compared to females for the 100 ppm exposure group (Table 1). DCVG cleared systemic circulation in a first-order manner quickly after cessation of the Tri exposure. The $t_{1/2}$ values for systemic clearance of DCVG were similar between genders (0.74 and 0.94 h for males and females, respectively).

These results clearly demonstrate that the GSH conjugation pathway is functional in healthy, human subjects and that the initial, stable metabolite can be detected at micromolar concentrations in the blood. An additional marker of the GSH conjugation pathway has been the recovery of one of the stable end-products of the pathway, the mercapturates NAc-1,2-DCVC and NAc-2,2-DCVC, in the urine. Despite development of a sensitive GC/MS assay with a limit of detection of 50 ng/ml, the mercapturates were not consistently detected in the urine of human subjects exposed to 100 ppm Tri. NAc-2,2-DCVC was only consistently

TABLE 1. Pharmacokinetic Parameters of DCVG in Blood of Males and Females Exposed to 100 ppm Tri

Sex	$t_{1/2}$ (h)	AUC (nmol DCVG/ml blood/h)
Males (n = 8)	0.74	153.9
Females (n = 8)	0.94	45.7

Note. Healthy human volunteers were exposed to 100 ppm Tri by inhalation for 4 h and DCVG formation was monitored in blood samples obtained over the course of several hours after the exposure. Data on DCVG content in blood (cf. Figure 3) were analyzed to obtain pharmacokinetic parameters, which are summarized here.

found in the urine of one subject of the eight males exposed to the higher dose of Tri. A maximal content of NAc-2,2-DCVC of 74 ng/ml was found 32 h after the end of the Tri exposure period. Interestingly, this subject also exhibited one of the highest maximal contents of DCVG in the blood (94 nmol/ml, or double the mean maximal content).

Because the HPLC method that we used to measure DCVG also allows for measurement of GSH and related thiols and disulfides as well as glutamate, several of these compounds were measured in the blood of subjects exposed to Tri. Furthermore, increases in excretion of GSH or related compounds can be an indicator of renal injury or alterations in renal GSH metabolism. Contents of glutamate, cystine, and cysteine in the blood exhibited considerable variability among subjects and with time (data not shown). Glutamate content in blood averaged nearly 50% higher in females than in males, with males exposed to 100 ppm Tri exhibiting values over the 4 d of urine collection of 38.0 ± 5.4 to 52.0 ± 9.9 nmol glutamate/ml blood (n = 8) and females exposed to 100 ppm Tri exhibiting values over the 4 d of urine collection of 52.6 ± 4.6 to 79.5 ± 11.0 nmol glutamate/ml blood (n = 8). No consistent differences in cystine were observed between males and females, with males exposed to 100 ppm Tri exhibiting values over the 4 d of urine collection of 20.8 ± 3.5 to 29.8 ± 5.9 nmol cystine/ml blood (n = 8) and females exposed to 100 ppm Tri exhibiting values over the 4 d of urine collection of 25.1 ± 3.5 to 41.0 ± 9.0 nmol cystine/ml blood (n = 8). Blood content of cysteine exhibited much more variability: Males exposed to 100 ppm Tri exhibited values over the 4 d of urine collection of 0.97 ± 0.32 to 2.50 ± 0.94 nmol cysteine/ml blood (n = 8) and females exposed to 100 ppm Tri exhibited values over the 4 d of urine collection of 0.56 ± 0.17 to 6.33 ± 4.77 nmol cysteine/ml blood (n = 8). In all cases, cystine accounted for >90% of total cysteine equivalents (i.e., cysteine + 2 cystine), which is consistent with the known low concentrations of cysteine both in plasma and in red blood cells and the high concentration of cystine in plasma (Lash & Jones, 1985b).

The content of the various low-molecular-weight forms of glutathione in blood, including GSH, GSSG, and CySSG, also showed some variability between subjects and with time (data not shown). Because tissue contents of GSH are known to exhibit a diurnal variation, it is not surprising that blood GSH content varied with time. The patterns of changes in blood GSH content in males and females were nearly identical, with maximum and minimum values in the blood of subjects exposed to 100 ppm Tri being obtained at the same measurement times. A peak in the content of GSH (413 ± 77 and 495 ± 101 nmol/ml blood in males and females, respectively; n = 8) was observed at 4 and 4.25 h (1:00 p.m. and 1:15 p.m. of d 1), a minimum that was markedly lower than values at other time points was observed at 16 h (1:00 a.m. of d 2; 261 ± 43 and 242 ± 38 nmol/ml blood in males and females, respectively; n = 8), and a lower

value at 94 h (7:00 a.m. of d 4; 343 ± 62 and 366 ± 93 nmol/ml blood in males and females, respectively; n = 8) than at the two previous time points were observed. Content of GSSG was 5% to 10% of that of GSH, and GSH and GSSG values generally varied in parallel with each other. GSSG content over the 4-d collection period varied between 16.4 ± 3.0 and 29.8 ± 6.3 nmol/ml blood in males (n = 8) and 13.4 ± 3.9 and 19.6 ± 4.2 nmol/ml blood in females (n = 8). Content of CySSG was <1% of that of GSH and showed no consistent pattern of variability with time. While mean values over the 4-d collection period for CySSG were generally higher in females (0.70 ± 0.22 to 1.79 ± 0.84 nmol/ml; n = 8) than in males (0.50 ± 0.13 to 1.65 ± 0.84 nmol/ml; n = 8), there was considerable interindividual variability.

A similar time-dependent pattern that was independent of sex was observed for the content in blood of total GSH equivalents (i.e., GSH + CySSG + 2 GSSG). Males exposed to 100 ppm Tri exhibited values over the nearly 4 d of blood sampling of 308 ± 41 to 505 ± 94 nmol total GSH-equivalents/ml blood (n = 8), and females exposed to 100 ppm Tri exhibited values over the 3 d of blood sampling of 275 ± 36 to 527 ± 14 nmol total GSH-equivalents/ml blood (n = 8). The redox state of GSH in blood was relatively constant with time and did not differ between males and females. The percent GSH in blood, calculated as the ratio of GSH to the sum of all GSH equivalents (i.e., GSH + CySSG + 2 GSSG) × 100%, varied between 85% and 93% over the 4 d.

Contents of urinary GSH varied over a wide range among subjects and with time, with values of less than 1 μmol to more than 6 μmol total GSH excreted at any time (Tables 2 and 3 and Figure 4A). Because the variation was relatively large and the sample size was only eight, there was no statistically significant difference in individual pairs of urinary values for males and females at the same time point. Nonetheless, females exposed to 100 ppm Tri appeared to have higher urinary GSH content than males exposed to 100 ppm Tri at several of the later postexposure time points. Analysis of the data by an analysis of variance (ANOVA) showed that the influence of sex as an independent variable was statistically significant. There also appeared to be a general trend toward higher peaks in urinary GSH content over time. Summation of 24-h urinary GSH excretion over the 4 d of sampling, however, showed no significant differences between males and females or between values in each of the 24-h time periods (Figure 4B).

DISCUSSION

This report documents the finding of measurable amounts of DCVG in the blood of healthy, human volunteers who were exposed by inhalation for 4 h to either 50 ppm or 100 ppm Tri. These data provide direct, in vivo evidence for the function of the GSH conjugation pathway in humans.

TABLE 2. Urinary Content of GSH in Males Exposed to 100 ppm Tri

Time after start of Tri exposure (h)	Time of day	Total urine GSH (μmol)	*n*
−2.25 to −0.75	Day 1: 6:45 to 8:15 a.m.	1.08 ± 0.43 (0.36, 3.58)	7
3 to 4.75	12:00 to 1:45 p.m.	4.71 ± 1.28 (1.22, 11.06)	8
5	2:00 p.m.	0.46 ± 0.09 (0.25, 0.73)	5
6	3:00 p.m.	1.30 ± 0.45 (0.57, 4.24)	8
8	5:00 p.m.	1.55 ± 0.43 (0.40, 4.07)	8
10 to 11.5	7:00 to 8:15 p.m.	1.43 ± 0.28 (0.79, 2.34)	6
12	9:00 p.m.	1.23 ± 0.37 (0.20, 2.67)	6
14	11:00 p.m.	1.85 ± 0.93 (0.34, 7.28)	7
16	Day 2: 1:00 a.m.	2.38 ± 1.23 (0.38, 10.7)	8
18	3:00 a.m.	1.00 ± 0.23 (0.33, 2.18)	7
20	5:00 a.m.	1.34 ± 0.32 (0.37, 2.81)	8
22	7:00 a.m.	1.52 ± 0.48 (0.30, 3.30)	7
23.25 to 26	8:15 to 11:00 a.m.	4.12 ± 2.17 (0.14, 16.3)	7
28 to 31.5	1:00 to 4:30 p.m.	4.94 ± 1.99 (0.77, 14.3)	7
32 to 35.75	5:00 to 8:45 p.m.	3.40 ± 1.22 (0.51, 9.18)	8
36.75 to 38.75	9:45 to 11:45 p.m.	1.83 ± 1.06 (0.30, 4.95)	4
40 to 45	Day 3: 1:00 to 6:00 a.m.	2.31 ± 0.80 (0.60, 5.47)	7
45.5 to 49.75	6:30 to 10:45 a.m.	4.60 ± 1.92 (0.58, 10.4)	5
50 to 53.75	11:00 a.m. to 2:45 p.m.	2.11 ± 0.94 (0.33, 5.47)	6
54 to 58.75	3:00 to 7:45 p.m.	3.09 ± 1.51 (0.19, 11.5)	7
59.5 to 62.5	8:30 to 11:30 p.m.	1.33 ± 0.35 (0.49, 3.21)	7
63.75 to 69.5	Day 4: 12:45 to 6:30 a.m.	5.88 ± 2.14 (1.13, 16.4)	7
70.25 to 74.5	7:15 to 11:30 a.m.	5.49 ± 1.78 (1.24, 11.9)	6
76 to 79.5	1:00 to 4:30 p.m.	4.32 ± 1.51 (1.43, 8.47)	4
80.25 to 84.75	5:15 to 9:45 p.m.	6.77 ± 3.56 (0.23, 24.1)	6

Note. Healthy, human male volunteers were exposed to 100 ppm Tri for 4 h. Urine samples were collected at the indicated times. GSH content of urine was analyzed as *S*-carboxymethyl-*N*-dinitrophenyl derivatives by HPLC. Results are means ± SE of the indicated number of measurements. Values in parentheses are the maximum and minimum values.

The additional finding of markedly higher amounts of DCVG in the blood of males as compared with females has potentially important implications regarding the role of the GSH conjugation pathway in the generation of toxic metabolites from Tri and in the risk to humans from nephrotoxicity or nephrocarcinogenicity due to Tri exposure.

Although flux through the P-450 pathway is quantitatively greater than that through the GSH pathway (Davidson & Beliles, 1991; Goeptar et al., 1995), maximal levels of DCVG in the blood of these subjects were similar to those reported in the parallel study of P-450-derived metabolites of Tri in the blood from these same human volunteers (Fisher et al., 1998). Maximal levels of trichloroacetate and trichloroethanol in the blood of males exposed to 100 ppm Tri were approximately 7 to 10 μg/ml and 7 μg/ml, respectively. Conversion of the values reported here from nanomoles to micrograms shows that maximal levels of DCVG in the

blood of males exposed to 100 ppm Tri were approximately 8 to 10 μg/ml. A major difference in the recovery of the oxidative metabolites and DCVG, however, is that recovery of DCVG in blood was transient, reaching a maximum at 2 to 4 h and being completely eliminated from blood by 12 h. Trichloroacetate levels in blood reached maximal levels at approximately 20 h and maintained these levels up to 96 h, while trichloroethanol levels in blood were more similar to those of DCVG, reaching maximal levels at 6 to 8 h and being nearly completely eliminated by 24 h. Hence, the AUC values for both of the major oxidative metabolites were markedly higher than those for DCVG.

Analysis of the individual values for DCVG content in blood rather than mean values for each sex suggested the possibility that two subpopulations of individuals exist, one that forms DCVG in high amounts and one that forms DCVG in low amounts. One possible explanation for this is that a genetic polymorphism exists in the enzyme or enzymes that form DCVG. Interpretations should be made cautiously, however, because sev-

TABLE 3. Urinary Content of GSH in Females Exposed to 100 ppm Tri

Time after start of Tri exposure (h)	Time of day	Total urine GSH (μmol)	*n*
−3 to −1	Day 1: 6:00 to 8:00 a.m.	2.17 ± 0.93 (0.03, 5.48)	6
2.25	11:15 a.m.	(0.86, 5.48)	2
4.5	1:30 p.m.	4.10 ± 1.46 (0.41, 11.9)	8
5	2:00 p.m.	0.57 ± 0.17 (0.04, 1.44)	7
6	3:00 p.m.	1.48 ± 0.39 (0.06, 3.71)	8
8	5:00 p.m.	1.85 ± 0.35 (0.97, 3.46)	7
10	7:00 p.m.	1.36 ± 0.34 (0.21, 2.96)	8
12	9:00 p.m.	1.81 ± 0.36 (0.50, 3.43)	8
14	11:00 p.m.	2.04 ± 0.43 (0.55, 3.95)	8
16	Day 2: 1:00 a.m.	1.97 ± 0.56 (0.50, 4.64)	7
18	3:00 a.m.	1.90 ± 0.50 (0.42, 4.36)	8
20	5:00 a.m.	1.69 ± 0.42 (0.45, 3.52)	7
22	7:00 a.m.	2.02 ± 0.60 (0.34, 3.75)	6
24.5 to 27	9:30 a.m. to 12:00 p.m.	3.90 ± 1.04 (0.23, 6.56)	6
28.5 to 32.5	1:30 to 5:30 p.m.	3.58 ± 1.05 (0.19, 5.79)	6
32.75 to 38.25	5:45 to 11: 15 p.m.	4.40 ± 1.27 (0.69, 10.4)	8
40 to 43.75	Day 3: 1:00 to 4:45 a.m.	2.59 ± 1.29 (0.07, 7.28)	5
44.75 to 49.75	5:45 to 10:45 a.m.	5.99 ± 2.09 (0.09, 18.1)	8
50.25 to 55.75	11:15 a.m. to 4:45 p.m.	2.25 ± 0.63 (0.10, 4.28)	7
56.25 to 63.5	5:15 p.m. to 12:30 a.m.	6.52 ± 1.84 (0.16, 13.9)	7
66.25 to 73.25	Day 4: 3:15 to 10: 15 a.m.	6.86 ± 2.41 (0.30, 20.3)	8
74 to 78.25	11:00 a.m. to 3:15 p.m.	4.56 ± 1.44 (0.12, 9.04)	6
80.25 to 85.75	5:15 to 10:45 p.m.	7.31 ± 1.97 (0.34, 17.3)	8

Note. Healthy, human female volunteers were exposed to 100 ppm Tri for 4 h. Urine samples were collected at the indicated times. GSH content of urine was analyzed as *S*-carboxymethyl-*N*-dinitrophenyl derivatives by HPLC. Results are means ± SE of the indicated number of measurements for $n \geq 3$. Values in parentheses are the maximum and minimum values or are individual values for $n < 3$.

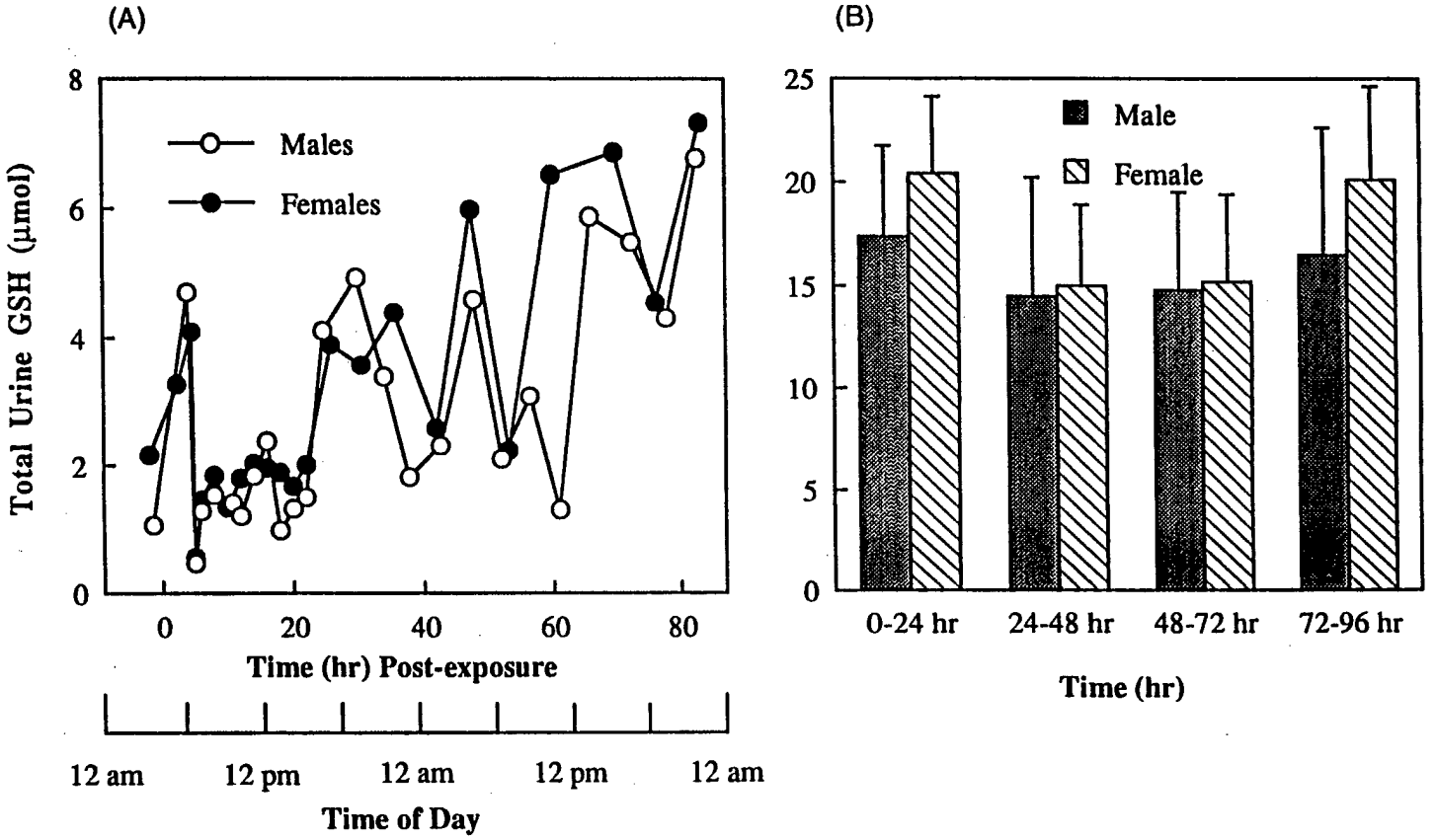

FIGURE 4. Time course of urinary excretion of GSH in males and females exposed to 100 ppm Tri. (A) Data from Tables 1 and 2 are plotted to show the complete time course of total urinary GSH content in male and female subjects exposed to 100 ppm Tri for 4 h. Results are means of measurements from five to eight individuals. A second *x* axis, showing time of day, is also shown to illustrate the diurnal variation in GSH excretion. Standard error values, which are omitted for clarity, are given in the tables. (B) Data over the four 24-h intervals of sample collection were pooled. Results are means ± SE of measurements from eight subjects each for males and females.

eral steps occur that determine how much DCVG is recovered in the blood. These steps include the initial conjugation of Tri with GSH that is catalyzed by GSTs and occurs primarily in the liver, efflux of the DCVG from liver into plasma or bile, enterohepatic circulation, and renal extraction. Individual variation in any one of these steps or processes could markedly alter the amount of DCVG recovered in the blood. Although there does appear to be a sorting of DCVG contents into a high and a low group, most of the individuals belonging to the high group were male and most of those belonging to the low group were females, and the males exhibited a 3.4-fold greater AUC than the females. Inasmuch as a relatively small sample size was used (i.e., 8 males and 8 females for the 100 ppm Tri exposure), additional sampling could help clarify this issue.

Besides the initial metabolite, DCVG, other metabolites that are derived from the GSH conjugation of Tri and that may theoretically be recovered in either blood or urine include DCVC, NAc-1,2-DCVC, and NAc-2,2-DCVC. Although, one might expect to recover some small amount of DCVC in blood, none was detected in the present study. This is likely due to the predominance of DCVC formation in and the rapid extraction of DCVG by the kidneys.

Because of the difficulty in detecting β-lyase-generated metabolites of DCVC, most researchers have relied on measurements of urinary mercapturate excretion to provide an indication of flux of Tri through the GSH conjugation pathway. The absence of consistent detection of NAc-1,2-DCVC or NAc-2,2-DCVC in the urine of the human volunteers in this study is not really surprising considering the relatively low amounts of DCVG that were measured in the blood. By the time the blood-borne DCVG is extracted and further metabolized by the kidneys, there are other fates besides mercapturate formation and urinary excretion. This would lead to a dilution over time in the amounts of mercapturate excreted. Additionally, another reason for the absence of consistent detection of mercapturates is that other metabolites may have formed, including those that may undergo fecal excretion, and these were not quantitated in the present study.

Controversy regarding the kidneys as a target organ of Tri in humans has centered primarily around four points: (1) There have been inconsistent observations of renal tumors in rats and these have been observed predominantly in male rats, suggesting that these tumors may be a male rat-specific response, analogous to the accumulation in kidneys of male rats of $\alpha_{2\mu}$-globulin after exposure to certain halogenated hydrocarbons (Lehman-McKeeman et al., 1990). (2) Rates of Tri metabolism by the GSH conjugation pathway are much slower than those by the P-450 pathway. (3) β-Lyase activity in human kidney is much lower than that in rat kidney (Lash et al., 1990). (4) Markedly higher amounts (100- to 4000-fold) of P-450-derived metabolites of Tri than of mercapturates are recovered in the

urine of either rats or humans treated with or exposed to Tri, suggesting that the GSH conjugation pathway is quantitatively minor for Tri.

The first point is beyond the scope of the present study. It should be noted, however, that the incidence of renal tumors is extremely rare, so that even when a small but statistically significant increase in renal tumors is observed, further study is warranted. Regarding the relative flux of Tri through either P-450 or GSH conjugation, it is difficult to interpret direct comparisons of reaction rates because the two pathways generate metabolites that are very different chemically. Specifically, metabolites of Tri generated by the oxidative pathway (i.e., trichloroethanol, trichloro- and dichloroacetate, chloral hydrate, etc.) are chemically stable, whereas the nephrotoxic and nephrocarcinogenic metabolite generated by the action of the β-lyase on DCVC is chemically reactive and unstable. This reactivity raises two issues: First, such a reactive and unstable metabolite may produce a disproportionately high degree of toxicity per mole as compared to a chemically stable metabolite; second, the chemical reactivity of DCVSH makes it difficult to accurately quantitate flux through this pathway, as discussed earlier. This leads into the final point, namely, that of the comparison between urinary excretion of mercapturates and P-450-derived metabolites. Measurements of NAc-1,2-DCVC and NAc-2,2-DCVC do not reflect conversion of DCVC to the nephrotoxic and nephrocarcinogenic species. Rather, the mercapturates are the detoxication products of DCVC. Therefore, the only valid use for data on recovery of mercapturates in the urine is as an indicator of exposure. These data cannot be used quantitatively to make conclusions about GSH-dependent bioactivation of DCVC.

Analysis of the other blood and urine data did not reveal any effects that appeared to be due to the Tri exposure. However, these data provide important baseline data on GSH status in these individuals and provide novel examples of sex-dependent differences in levels of these compounds. Blood GSH content showed a time-dependent variation that was similar in both males and females. The nearly twofold variation in the concentration of GSH in the blood over time is consistent with the known diurnal variation in tissue GSH and is not due to hematological changes, edema, or renal insufficiency. Urinary GSH content also exhibited time-dependent variation, although when total GSH excretion over each 24-h period was compared over each of the four days of sampling, no differences were observed between sexes or over time (cf. Figure 4B). However, the apparent trend toward increased maximal urinary GSH excretion at later postexposure time points can be an indicator of potential renal injury. If such injury did occur, however, it was minor and transient.

In summary, this study gives direct evidence of GSH conjugation of Tri in human volunteers exposed to Tri. Importantly, we determined amounts of a GSH-derived metabolite that is proximal to DCVC in the metabolic pathway. This is critical because the only other published evidence of GSH

conjugation of Tri in humans comes from data on mercapturate excretion (Bernauer et al., 1996; Birner et al., 1993), and the relevance of mercapturate levels to renal toxicity is uncertain. Males exhibited markedly higher contents than females of DCVG in the blood, suggesting that their potential risk to Tri-induced renal toxicity, whatever it may be, could be greater than that of females.

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